CCLXXXI. STUDIES OF THE ESSENTIAL UNSATURATED FATTY ACIDS IN THEIR RELATION TO THE FAT-DEFICIENCY DISEASE OF RATS

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The experiments of Burr & Burr [1929; 1930] and of Burr *et al.* [1932] clearly established that the complete exclusion of fat from a diet containing all known accessory factors slowly produced in young rats a definite disease. Its symptoms were cessation of increase in length and weight with development of a dry scurfy skin, scaly tail, irregular ovulation and lesions of the kidney and urinary tract. The rats receiving the fat-free diet ate as much food and drank twice as much water as their controls, but notwithstanding became extremely emaciated. At 4 months after weaning their weight reached a level of about 150 g. and then remained stationary, but weight increase was resumed when 10 drops daily of the fatty acids from lard were added to the diet. The curative effect of the dose was immediate and the gain in weight was quite disproportionate to the calorie value of the curative material administered. Various other oils were tested and the conclusion was drawn that their curative potencies appeared to be correlated with their linoleic acid contents. Burr and Burr therefore suggested that the rat is unable to synthesize linoleic acid and that this acid is essential for its normal growth. Linolenic acid was also examined and was reported to be equal to linoleic acid in curative effect. The full literature of this subject has recently been reviewed by Anderson & Williams [1937] to whose excellent account the reader is referred. Work which is particularly relevant to the present study will be referred to as the various materials tested are discussed.

There is no evidence as to the part played in metabolism by these unsaturated fatty acids. Linoleic acid is present chiefly in the fatty acids of the phospholipins and to a less extent in the neutral fat. Linolenic acid, on the other hand, appears to be changed immediately on entering the body and is not found even in small amounts, unless when large quantities have been administered. It is known, owing chiefly to the work of Sinclair [1929–30], that the highly unsaturated acids are held in the phospholipins with extreme tenacity and are eliminated only very slowly when animals are transferred to a diet from which the acids are absent. They do not appear to represent stages in the combustion of the fatty acids, but to have some unknown significance in the body metabolism. It seemed possible that an oxidation product might be formed as a further stage in some metabolic process and it appeared desirable to compare the effect of the unsaturated acids with that of some closely related oxidation products.

The present investigation has, therefore, included the following:

(1) A reproduction of the work of Burr and Burr with a study, as far as possible quantitative, of the relative potencies of linseed oil, linoleic and linolenic

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acids, both to restore weight increase and to alleviate skin lesions: a test of the potencies of the elaidized linoleic and linolenic acids compared with those of the original products.

(2) A test of the potencies of certain oxidation products of linoleic and linolenic acids.

(3) A comparison of the activities of fractions of the unsaturated acids from lard and from linseed oil.

(4) A comparison of the activities of linseed oil and raisin seed oil.

(5) An examination of the potency of the methyl ester of the highly unsaturated docosahexaenoic acid ($C_{22}H_{32}O_2$) of cod liver oil. Through the generosity of Dr E. H. Farmer an opportunity was afforded to test the efficacy of this acid recently isolated by him from cod liver oil [Farmer & Van den Heuvel, 1938, 2].

(6) A test of the curative actions, if any, of chaulmoogra oil and chaulmoogric acid, of methyl arachidate and of 9:10:12-trihydroxystearic acid.

Biological technique

The experiments described in the present paper have been in progress for about 5 years so that the technique has varied slightly over this period, but the regime of Burr *et al.* [1932] has been followed fairly closely.

Young rats weaned on a stock diet at an age of 21 days and a weight of about 40 g. were placed in separate cages with coarse wire grids. Sometimes fine wire grids and cotton-wool bedding were given when the animals seemed in a precarious condition in severe weather. As it takes months to prepare the animals it is very important not to lose them when the time for testing approaches.

The diet consisted of: casein (British Drug Houses, fat- and vitamin-free), 12%; sucrose, 84·1%; salt mixture, McCollum 185 [McCollum & Davis, 1914], 3·9%.

The diet was given *ad libitum*, slightly moistened. The vitamin B complex was supplied as 0·65 g. per head daily of dried brewer's yeast which had been continuously extracted with ether for 24 hr. This supplement was given separately and was frequently refused at first, but if it was withheld for the first 7–10 days it was subsequently taken with avidity and was found an admirable vehicle for administering the test dose, whether this took the form of an oil or a solid. Vitamins A and D were given as 0·5 mg. daily per head of the unsaponifiable fraction of a cod liver oil concentrate, of value 4000 blue units, dissolved in paraffin oil. In the latest experiments (rats belonging to litters 4273–4350) vitamin A was derived from a fish liver oil concentrate, but vitamin D was given as 2–3 I.U. daily of irradiated ergosterol dissolved as before in paraffin oil. Vitamin E was not given except in the latest experiments (rats numbered as above) when it was given for a portion of the depletion period and for the test period as an unsaponifiable fraction of wheat germ oil, prepared by the Glaxo Laboratories. It was administered without solvent in a dose equivalent to about 2·0 g. wheat germ oil weekly. The distilled water used for drinking and for moistening the food contained 0·27 mg. KI per l.

On this diet the behaviour of the rats conformed with that described by Burr & Burr. After about 2 months the weight curve began to flatten and the skin lesions began to appear and to become progressively more severe. Tests were usually started after about 4 months on the diet, but no rigid rule was established in this respect. A few tests which were started when the rats had only been receiving the deficient diet for about 70 days could not be regarded as reliable, at

least from the standpoint of resumption of weight increase. After a period of 4 months or more on the deficient diet the writers considered that weight increase could be accepted as some measure of the potency of the materials administered and the total weight increase in an experimental period of 35 days was taken as one criterion. The total weight increase was calculated as the weight on the day on which dosing was begun subtracted from the weight 35 days later.

For quantitative estimation, weight increase has been the chief criterion used by Burr and his co-workers, by Moore [1937] and by Turpeinen [1938]. The last also used as criterion the restoration of the oestrous cycle towards normal.

While accepting weight increase as one criterion, the present workers hold very strongly the opinion that, since weight increase is a non-specific response, it should not be used as the only criterion, and that cure of symptoms must be included as an indispensable part of the test. A serious objection to the use of this additional criterion is the great irregularity with which most of the lesions tend to appear. The kidney symptoms described by Burr & Burr were sometimes seen. The development of the so-called scaly tail, better described as a corrugated or annulated tail, was very irregular, the tails of individual rats sometimes being almost normal at a time when their litter-mates were showing a very severe degree of annulation. The other symptoms observed were general dryness of the skin and thinness of the hair with much scurf, showing best on parts of the body where the fur was dark. Dryness and scurfiness of the fore and hind paws and ears were also present; the most regularly observed site for this state was the dorsal surface of the hind feet and front of the ankles. Dryness here occurred invariably and was finally adopted as the most satisfactory criterion for studying the cure of skin symptoms. With potently curative materials the skin of the insteps could be restored to normal within the 35 days of the experimental period during which curative material was administered and weight increase measured. Only three degrees of healing could be satisfactorily distinguished: complete healing, designated by $++$ in the Tables, in which the ankles were restored to normal; partial healing, designated by $+$, in which improvement was definitely present but the ankles were still abnormal; no healing, designated by θ, in which the ankles were unchanged or worse.

Even when lesions of the tail were well developed, as they usually, but not always, were, it was found impossible to use them to measure the rate of healing since the process was much too slow. Within the experimental period of 35 days, even when healing of the ankles was complete, the tail might show little change. If the cure was continued long enough the tail could be restored entirely to normal, but in 35 days it might even appear worse owing to the vigorous desquamation which sets in at the beginning of the healing process. The writers share the impression expressed by Brown & Burr [1936] that the skin lesions are somewhat influenced by weather changes, being aggravated by the same type of cold dry weather as causes the human skin to chap. This adds to the difficulties in appraising cure. Very severe lesions of the external skin about the mouth of the rats were sometimes observed but these were due to the burning action of the curative materials administered and not to the deficiency. For instance, methyl linoleate and linolenate, and the methyl docosahexaenoate in these experiments, and ethyl laurate and acids from hydrogenated tung oil in other experiments, had the effect of seriously damaging the skin about the mouth, causing the upper layers with all the hair attached to shell off. No internal lesions appeared to have been produced. Materials having this effect were therefore always intimately mixed with the dried yeast dose, and in this way did not wet the rat's lips and were not objected to.

Although a source of vitamin E was given in some experiments and not in others there was no convincing evidence that its absence made any significant difference in this test. Skin lesions were healed and weight increase appeared to be promoted as well in its presence as in its absence. A quite different type of skin lesion with large raw and bleeding areas appeared in some cases and may have been due to vitamin E deficiency but this is quite uncertain. All the rats tended to become very nervous and fidgety when untreated and to improve on treatment, so that the essential unsaturated fatty acid was the probable sedative factor.

Since there is no recognized standard material and no curve of response available for tests on the unsaturated fatty acids, estimations of the potency of materials were made as a series of simultaneous comparisons in which the weight increase and degree of healing of the skin of the ankles of litter-mate rats were compared. Some negative controls and some positive controls receiving linseed oil were usually included, but the larger number of rats was devoted to some special comparison. Thus, for example, in the first experiment the action of methyl linoleate was directly compared with that of methyl linolenate; in the second the potencies of linusic and *iso*linusic acids were compared with one another and with that of linseed oil. When only small amounts of material were available or when preliminary experiments gave an unequivocally negative result, full comparisons were not made. A certain number of materials tested in this way are included in Table III.

Since rats for this test take so long to prepare they were sometimes used a second time when the material first tested had proved negative. Negative controls were also subsequently used for tests. As an additional form of positive control, rats which had given a negative result were usually eventually treated with some known positive material such as wheat germ oil or linseed oil, but no record of this form of positive control is included here.

Experimental

(1) *Comparison of the relative potencies of methyl linoleate, methyl linolenate and linseed oil with one test of methyl β-linoleate. Effect of elaidization on the potency of linoleic and linolenic acids.*

Burr *et al.* [1932] found methyl linoleate and methyl linolenate equally effective in curing the lesions and restoring weight increase in rats which had received a fat-free diet. The curative effect of linoleic acid has been repeatedly confirmed [Evans & Lepkovsky, 1932; Tange, 1932; Becker, 1934; Becker, 1935; Moore, 1937; Turpeinen, 1938]. As regards linolenic acid the facts are not so well established. The experiments with pure methyl linolenate seem to have been repeated only by Tange [1932], who concluded that linoleic and linolenic acids were equally effective in promoting weight increase and bringing about a cure. His experiments do not, however, appear entirely to justify this conclusion. He describes experiments in which groups of 2 and 3 rats were dosed daily with one drop of methyl linoleate and linolenate respectively, and says that when growth was somewhat retarded the dose of methyl linolenate was increased to 2 drops but no great improvement was observed; he further reports that this retardation and irregularity, which were not shown with linoleic acid, seemed to be rather influenced by heat. A chart shows as equal the growth curves of two rats dosed with methyl linoleate and of two dosed with methyl linolenate, but no details are given as to the size of the doses.

Since linolenic acid is always accompanied in plant oils by linoleic acid,

it is essential that the linolenic hexabromide from which the linolenic acid is prepared should be carefully purified. In the experiments of Burr & Burr the samples of linoleic and linolenic acids used were obtained from maize and linseed oils respectively. The unsaturated acids were brominated and the resulting tetra- and hexa-bromostearic acids were carefully purified by recrystallization. These were then debrominated, esterified and distilled at less than 1 mm. pressure. The preparation of linolenic hexabromide melted at 180 to 181°, so that it could not have contained any appreciable quantity of the linoleic tetrabromide. Burr & Burr tested the material thus prepared on a group of 3 rats, each receiving 3 drops of methyl linolenate daily, and on a group of 2 rats, each receiving 3 drops of methyl linoleate daily. The average total weight increases of the two groups in 63 days were almost identical, being respectively 31 and 31·5 g.

Chemical preparation. In the present experiments two preparations of linolenic hexabromide, derived from linseed oil and melting at 180–182° and at 182·5° respectively, were used for the preparation of methyl linolenate; the Br contents were 62·0 and 63·06 % for the two samples, the theoretical value for linolenic hexabromide being 63·3 %. The iodine values of the two specimens of methyl linolenate used in the feeding tests were 230·6 and 245 respectively.

The linoleic acid prepared from maize oil was converted into the linoleic tetrabromide, the M.P. of which was 115°; the Br contents were 54·4 and 53·7 %, the theoretical value being 53·3 %. This material was debrominated and esterified, and the methyl linoleate thus prepared gave iodine values of 165·6 and 166·5. To avoid oxidation the unsaturated esters were sealed in small tubes filled with nitrogen and kept at 0°. When a tube was in use for feeding the I.V. was tested at intervals to see that no marked oxidation had taken place. Under these conditions the I.V. of the methyl linoleate had fallen after 24 days from 166·5 to 163·1 and that of the methyl linolenate from 245 to 240.

A sample of methyl β-linoleate was prepared by debrominating the liquid form of linoleic tetrabromide. It had an iodine value of 135·2.

The sample of linseed oil used for comparison in the feeding tests had I.V. 180 and contained about 26 % linoleic and about 44 % linolenic acid.

Biological tests. The biological tests were essentially a comparison between the potencies of methyl linoleate and methyl linolenate. Groups of 7–9 rats received a daily dose of 6 drops (0·08 g.) of one of these two substances or served as negative controls with no addition (see Tables I *a* and *b*). The average total weight increase in 35 days for the group receiving methyl linoleate was 23 g., for that receiving methyl linolenate 8 g. and for the negative control group 2 g. As regards the skin lesions of the ankles, all but one of the rats receiving methyl linoleate showed complete healing in 35 days; of those receiving methyl linolenate all showed partial, but none complete, healing in the period, and the negative control group showed no healing at all.

In addition two animals received daily 5 drops (about 0·08 g.) of linseed oil, one received 6 drops (0·08 g.) of methyl β-linoleate and four received 1 drop (0·013 g.) of methyl linoleate. The numbers of rats for these tests are small but reference to Tables I *a* and I *b* shows that the average behaviour of these groups both as regards cure of lesions and resumption of weight increase was intermediate between that of the group receiving 0·08 g. methyl linoleate, and that receiving 0·08 g. methyl linolenate. The average weight increase of 14 g. shown by the rats receiving one drop of methyl linoleate was greater than that of 8 g. shown by the rats receiving 6 drops of methyl linolenate.

The behaviour of one rat was of particular interest. After having received 6 drops methyl linolenate daily for 13 days it had lost 19 g. in weight and was in

Table I*a*. *Total wt. increase in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given methyl linoleate, methyl β-linoleate, methyl linolenate, no supplement or linseed oil*

(Results marked with the same symbol *, † etc., apply to the same individual rat in two different tests.)

Litter no.	Sex	Methyl linoleate 0·013 g. daily (g.)	Methyl linoleate 0·08 g. daily (g.)	Methyl β-linoleate 0·08 g. daily (g.)	Methyl linolenate 0·08 g. daily (g.)	No supplement negative controls (g.)	Linseed oil 0·08 g. daily (g.)
4330	♂	17*	13	.	.	−10*	.
	♀♀	.	.	.	14†	− 1	.
	♀	16	.	.	7†	.	.
4329	♂	.	19	.	2	−17	20
	♀♀	.	30	.	9	2‡	20
	♀	12‡	.	.	.	.	.
4338	♀	.	31	16	8	5§	.
	♀	.	18	.	−19‖ (13 days)	.	.
	♀	13§	39‖	.	25	.	.
4350	♂	.	.	.	3	3	.
	♀	.	10	.	3	5	.
Av.		14	23	16	8	2	20

Table I*b*. *Healing of skin symptoms in 35 days. Same series as above. Three degrees of healing recognized: complete* ++, *partial* +, *none* θ

Litter no.	Sex	Methyl linoleate 0·013 g. daily	Methyl linoleate 0·08 g. daily	Methyl β-linoleate 0·08 g. daily	Methyl linolenate 0·08 g. daily	No supplement negative controls	Linseed oil 0·08 g. daily
4330	♂	++*	++	.	.	θ*	.
	♀♀	.	.	.	+†	θ	.
	♀	+	.	.	+†	.	.
4329	♂	.	++	.	+	θ	+
	♀♀	.	++	.	+‡	θ	+
	♀	+‡	.	.	.	.	.
4338	♀	.	++	+	+	θ§	.
	♀	.	++	.	θ‖ (13 days)	.	.
	♀	+§	+‖	.	+	.	.
4350	♂	.	.	.	+	θ	.
	♀	.	++	.	+	θ	.

a very precarious condition. The dose was changed to 6 drops daily of methyl linoleate; the rat at once ceased to lose weight and quickly began to gain, making a total weight increase of 39 g. in the 35 days immediately ensuing (see Table I*a*, *b*, rat 4338 ♀‖). The skin lesions of the ankles were not, however, completely cured within this period. Rats did not usually lose weight when receiving methyl linolenate, so that this is the only case in which so marked a curative action of methyl linoleate in contrast with methyl linolenate was demonstrated.

A certain number of the rats continued to receive the unsupplemented deficient diet after the end of the 35-day period of dosage with methyl linoleate or linolenate, and they were observed for irregular periods varying from 30 to 80 days. Their behaviour showed a powerful and sustained action of methyl

linoleate and a weak and transitory one of methyl linolenate. Of 5 rats receiving the former, all continued to gain in weight slowly and the unhealed lesions of skin and tail, excluding the ankles which were already healed, progressed steadily towards cure. Of 3 rats which received 6 drops of methyl linolenate, only one made any sustained weight increase and none showed any progress of the cure; the ankles, which were only partly healed at the end of the period of dosage, never showed any tendency to complete healing (see Table II).

Table II. *Subsequent behaviour of rats receiving the unsupplemented fat-free diet in the period immediately following a 35-day period of dosage with 0·08 g. daily of methyl linoleate or methyl linolenate*

Litter no.	Sex	Previous daily supplement	Length of period without supplement days	Total wt. increase in period without supplement g.	Behaviour of symptoms in period without supplement
4330	♂	Methyl linoleate 0·08 g.	80	33	All skin and tail lesions continuing to progress towards normal
4329	♂	,,	70	28	
4329	♀	,,	70	20	
4338	♀	,,	30	16	
4338	♀	,,	36	13	
4330	♀	Methyl linolenate 0·08 g.	45	0 (+7, −7)	No progress in healing of lesions. Partly healed ankles showing no improvement
4329	♀	,,	42	1 (+4, −3)	
4329	♂	,,	45	12	

All the experimental evidence thus obtained, therefore, went to indicate that methyl linoleate exercised a potent and prolonged action in counteracting the effects of a fat-free diet, while methyl linolenate had only a weak and transitory action.

Discussion. The difference thus established is of especial interest since there is general agreement that whilst linoleic acid is normally a constituent of the body lipins, linolenic acid is only present in small amounts, as the result of administering it in exceptionally large amounts [Ellis & Isbell, 1926; Spadola & Ellis, 1936]. Thus Hartley [1909] found evidence of the presence in the liver, heart and kidney of pigs of unsaturated acids with one, two, and four double bonds. Snider & Bloor [1932–3] found oleic, linoleic and arachidonic acids in ox liver lecithin, and failed to find linolenic acid, a result confirmed by Klenk & Schoenebeck [1932]. Turner [1930] found no linolenic acid in sheep's liver. Eckstein [1929] found linoleic and arachidonic acids in rat tissues but no evidence of linolenic acid. Levene & Rolf [1926] described linolenic acid as exceeding linoleic acid in amount in liver lecithin, but the hexabromide described, being soluble in ether, does not agree in properties with linolenic hexabromide. It seems certain, therefore, that though linolenic acid may be consumed in large quantities it is not taken up as a constituent of the body lecithins. Since it is not stored in the body it is not surprising that after its administration has been stopped, no further increase in weight or improvement of symptoms takes place in the rat. Linolenic acid must be more rapidly transformed on entering the body than in linoleic acid, and it is possibly owing to this change that methyl linolenate is so markedly inferior to methyl linoleate in its effect in counteracting the effects of a fat-free diet.

Effects of elaidized linoleic and linolenic acids. Tange [1932] added 0·5% elaidic acid to the fat-free diet of 5 rats of which two failed to show weight increase.

He reports that this acid was ineffective in curing symptoms and behaved like oleic acid.

Preparation. Linoleic and linolenic acids were elaidized according to the directions of Griffiths & Hilditch [1932] and the methyl esters were used for the biological test. The esters were orange in colour; the I.V. of the original methyl α-linoleate was 165·6, and after elaidization it was 157·2. The I.V. of the original methyl α-linolenate was 230·6, and after elaidization it was 215.

Biological test. One rat received 0·08 g. daily of elaidized methyl α-linoleate and showed a weight increase of 24 g. in the experimental period, with partial healing of skin lesions. Another rat received the same amount of elaidized methyl α-linolenate and showed no weight increase but about the same degree of healing; it was found to be suffering from the chronic lung affection of rats which would affect adversely its capacity to put on weight (see Table III). The elaidized methyl α-linoleate still contained a large proportion of non-elaidized linoleate; the degree of healing was inferior to that previously observed with the same dose of methyl linoleate.

(2) *Examination of the potencies of certain oxidation products of linoleic and linolenic acids.* (*a*) *Tetrahydroxystearic acids: tests on mixtures of α- and β-, and of γ- and δ-tetrahydroxystearic acids.* (*b*) *Dioxidostearic acid.* (*c*) *Hexahydroxystearic acids: linusic and* iso*linusic acids.*

(*a*) *Tetrahydroxystearic acids.* Nothing is known as to the changes which linoleic and linolenic acids undergo in the body. It seemed possible that, as a further stage in metabolism, oxidation products might be formed. This possibility cannot be tested exhaustively even for the hydroxystearic acids, since few of them have been isolated. Thus eight racemic forms of tetrahydroxystearic acid may theoretically be derived from linoleic acid but only four of these are known; α- and β-tetrahydroxystearic acids, melting respectively at 154·7° and 171·3°, have been obtained by the action of $KMnO_4$ on linoleic acid in alkaline solution. The γ- and δ-forms were obtained by Nicolet & Cox [1922] by the addition of HOBr to linoleic acid and the conversion of the hydroxybromo-derivatives into the tetrahydroxy-acids. The γ-form melted at 144·5°, the δ-form at 135°.

Preparation. Mixtures of the 9:10:12:13-α- and β-tetrahydroxy-acids were prepared with M.P. 164–166°, and of the 9:10:12:13-γ- and δ-tetrahydroxy-acids with M.P. 145–146° by the above methods. These two mixtures were used for biological tests.

Biological tests. Three rats received 0·2 g. daily of the mixture of the α- and β-forms, and one rat received 0·2 g. of the mixture of γ- and δ-forms (see Table III), but no weight increase or amelioration of skin symptoms occurred in any case. Thus no indication of the activity possessed by linoleic acid was shown by these tetrahydroxy-addition products of it.

(*b*) *Dioxidostearic acid. Preparation.* Dioxidostearic acid was prepared by the method of Green & Hilditch [1935], by the oxidation of methyl linoleate with perbenzoic acid in chloroform solution and recrystallization from hot 70% alcohol. It melted at 78–79°.

Biological test. A daily dose of 0·2 g. of the preparation was added to the diet of one rat for 35 days. It showed no gain in weight and no healing of skin symptoms; a litter-mate negative control gained 7 g. in the same period (see Table III).

Table III. *Total wt. increase and healing of skin symptoms in young rats which had received a diet devoid of fat for about 120 days and then various supplementary materials for differing periods*

Litter no.	Sex	Material	Dose daily g.	Duration of dosing days	Wt. increase g.	Healing of symptoms
4329	♂	Elaidized methyl α-linoleate	0·08	35	24	+
4329	♀	Elaidized methyl α-linolenate	0·08	35	0 (pneumonia)	+
4075	♀	9:10:12:13-α- and β-tetrahydroxy-stearic acid	0·2	35	0	θ
4076	♀	,, ,,	0·2	35	0	θ
4330	♂	,, ,,	0·2	35	− 9	θ
4330	♂	9:10:12:13-γ- and δ-tetrahydroxy-stearic acid	0·2	35	− 9	θ
4338	♀	Dioxidostearic acid	0·2	35	− 3	θ
4350	♂	Methyl docosahexaenoate	0·06–0·1	35	24	? < +
4350	♀	,,	0·06–0·1	35	21	? < +
4273	♂	Chaulmoogra oil	0·16	13	− 5	θ
		Chaulmoogric acid	0·2	5	0	θ
4330	♂	Methyl arachidate	0·3	15	− 9	θ
4273	♀	9:10:12-trihydroxystearic acid	0·2	35	− 4	θ

(*c*) *Hexahydroxystearic acids*: *linusic and* iso*linusic acids*. Thirty-two racemic isomerides of the hexahydroxystearic acids derived from linolenic acid are theoretically possible; the racemic linusic and *iso*linusic acids obtained by oxidation with $KMnO_4$ of the unsaturated acids of linseed oil [Rollett, 1909] are the only ones known.

Preparation. Linusic acid, M.P. 196–197° and *iso*linusic acid, M.P. 172–174°, were prepared as above.

Biological tests. The results are set out in Tables IV *a*, *b*. Five rats received 0·2 g. daily of linusic acid; 7 received the same amount of *iso*linusic acid, and 2 received 0·1 to 0·13 g. daily of a mixture of both acids. All tended to show some weight increase, the 12 animals which received 0·2 g. daily giving an average total weight increase for the experimental period of 6 g., as compared with an average total weight increase of 3 g. for 7 litter-mate negative controls with no supplement, and of 20 g. for 5 litter-mate positive controls receiving 3–10 drops (0·05–0·16 g.) linseed oil daily. This performance appears to represent a definite though small weight increase by the rats receiving the hexahydroxystearic acids, not greatly inferior to that of rats receiving methyl linolenate, as already described (see Table I *a*). As regards the healing of skin lesions the rats receiving the hexahydroxystearic acids behaved like the negative controls. It is regrettable that rats receiving methyl linolenate were not included simultaneously in this comparison; when it was begun the inferiority of linolenic to linoleic acid had not been established and it was thought that the mixture of both these acids, represented by linseed oil, would be a satisfactory material for a positive control.

The faeces of the rats fed with the tetrahydroxy-acids in the previous experiment and the hexahydroxy-acids in the present experiment were large and pale in colour and obviously contained a large amount of the hydroxy-acids administered. Indeed it is doubtful how much of substances of such high melting points and so insoluble in water could be absorbed; the amounts absorbed can only have been a small fraction of the dose administered. The solubility in water of the hexahydroxy-acids is appreciably greater than that of the tetrahydroxy-acids, and possibly more of these may pass through the intestinal wall. For a part of the experimental period one rat received ethyl linusate and another ethyl

Table IV*a*. *Total wt. increase in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given linusic acid,* iso*linusic acid, no supplement or linseed oil*

Litter no.	Sex	Linusic acid 0·2 g. daily g.	*iso*Linusic acid 0·2 g. daily g.	No supplement g.	Linseed oil 3–10 drops daily (0·05 to 0·16 g.) g.
4075	♀	9	9	.	17
	♂	.	.	8	.
4076	♀	11	12	4	17
4273	♀	− 4*	− 1*	− 1	.
	♀	0*	.	.	.
	♂	.	−13	−13	25
4330	♀	.	4	− 1	.
4350	♀	6	8	8	10
3262	♀	.	7	4	25
		Linusic and *iso*linusic acids mixed 0·1–0·13 g.			
2907	♀	9		− 1	.
2931	♂	35†		6†	26†
Av.		5	6	3	20
		8			

* Experiment lasted 30 instead of 35 days.
† Experiment started on 75th instead of about 120th day.

Table IV*b*. *Healing of skin symptoms in 35 days. Same series as in Table IVa. Three degrees of healing recognized, complete* ++, *partial* +, *and none* θ

Litter no.	Sex	Linusic acid 0·2 g. daily	*iso*Linusic acid 0·2 g. daily	No supplement	Linseed oil 3–10 drops daily (0·05–0·16 g.)
4075	♀	θ	θ	.	++
	♂	.	.	θ	.
4076	♀	θ	θ	θ	+
4273	♀	θ*	θ*	θ	.
	♀	θ*	.	.	.
	♂	.	θ	θ	+
4330	♀	.	θ	θ	.
4350	♀	θ	θ	θ	++
3262	♀	.	θ	θ	+
		Linusic and *iso*linusic acids mixed 0·1–0·13 g.			
2907	♀	θ		θ	.
2931	♂	θ†		θ†	++†

* Experiment lasted 30 instead of 35 days.
† Experiment started on 75th instead of about 120th day.

*iso*linusate, instead of the corresponding acids, in the hope that the esters might be better absorbed, but weight increase was not favourably affected. An attempt to promote absorption was also made by adding about 0·25 g. daily of coconut oil simultaneously with the dose of 0·2 g. daily of linusic acid to the diet of one rat for a part of the experimental period, but without result.

The experiments with these hydroxy-derivatives cannot, therefore, be regarded as conclusive since there was no test of the amount absorbed. The small positive effect on weight increase observed in the case of linusic and *iso*linusic acid seems, however, even more convincing when it is remembered that a considerable proportion of the original dose was certainly excreted in the faeces.

(3) *Comparison of the potency of the unsaturated acids from lard and from linseed oil*

When Burr & Burr [1929] first described the deficiency disease produced by a fat-free diet they found that the condition was cured or prevented by the inclusion of 2 % lard in the diet. They referred to lard as one of the best curative fats and suggested that this may possibly be due to the presence of arachidonic acid in the lard fat. Later Burr *et al.* [1932] tested the efficacy of arachidonic acid by replacing 10 % of a mixture of the esters of linoleic and linolenic acids by methyl arachidonate and comparing the effect of 3 drops of the mixture with that of 3 drops of the original ester mixture. The mixture containing the arachidonate proved to be appreciably less active in promoting weight increase than the original mixture. Turpeinen [1938], on the other hand, estimated the effect on weight increase of pure methyl arachidonate and found it three times as potent as methyl linoleate. He does not, however, give any account of its healing effect on the skin lesions, though he found it potent to restore the oestrous cycle towards normal.

The amount of arachidonic acid in lard is usually less than 0·1 % [cf. Ellis & Isbell, 1926; Spadola & Ellis, 1936]; the sample used by Burr & Burr contained 0·06 % so that it does not seem probable that the potency of the lard depended to any great extent on its content of arachidonic acid. Its effect must, therefore, have been due chiefly to its content of linoleic acid. In the lard derived from maize-fed pigs used by Burr & Burr, the percentage of linoleic acid was 6·7. The percentage of linoleic acid in lard is extremely variable, being dependent on the previous diet of the pig. The lard of an animal fed with a ration containing oils rich in linoleic acid may contain as much as 20 % of linoleic acid, and when soya bean has been included in the food 35 to 36 % has been found [Banks & Hilditch, 1932; Ellis & Isbell, 1926; Ellis *et al.* 1931], but usually the linoleic acid content is below 10 %. Percentages varying from 23 to 70 are given for the linoleic, and from 18 to 49 for the linolenic, acid content of linseed oil, but since the proportions of the two acids vary inversely to one another, the iodine value of the oil is fairly constant. Burr & Burr [1929] and Burr *et al.* [1932] found lard less potent than linseed oil.

Preparation. The methyl esters of the unsaturated acid fraction were prepared from American lard, distilled *in vacuo*, and a fraction with I.V. 111·7 was used for the biological tests. This would correspond with a mixture of about 70 % oleic and about 30 % linoleic acids.

A fraction of the unsaturated acids from linseed oil was also prepared; it had I.V. 199 to 200 and contained approximately 20 % oleic, 30 % linoleic and 50 % linolenic acid. The linoleic acid content of the fraction derived from the lard was thus about the same as that of the fraction derived from the linseed oil, but the linseed oil fraction contained in addition nearly double the amount of linolenic acid.

Biological tests. Groups of 4 litter-mate rats received 3 to 5 drops (about 0·05–0·08 g.) daily of the fraction of unsaturated acids from lard and from linseed oils, two animals in each group receiving 3 and two 5 drops. The average

total weight increase was 29 g. in both groups, and all the animals showed partial healing of the ankles to about the same extent (see Table V). It is therefore concluded that the activities of these two fractions were about the same.

Table V. *Total wt. increase and healing of skin symptoms in 35 days of young rats which had received a diet devoid of fat for about 120 days, and were then given unsaturated fatty acid fractions from lard or linseed oil*

Litter no.	Sex	Linseed oil acids 3–5 drops (0·05–0·08 g.) g.	Lard acids 3–5 drops (0·05–0·08 g.) g.	Linseed oil acids 3–5 drops (0·05–0·08 g.)	Lard acids 3–5 drops (0·05–0·08 g.)
3262	♂	36	27	+	+
	♀	21	21	+	+
1255's	♂	40	48	+	+
1258's	♀	16	22	+	+
Av.		28	29	.	.

These experiments seem to afford additional confirmation of the great superiority of linoleic over linolenic acid as a curative agent, for the two fractions contained about the same amount of linoleic acid, but the linseed oil contained a large amount of linolenic acid in addition.

Lard and linseed oil vary so much in their linoleic acid contents that considerable discrepancies between the results of different workers must be expected, and feeding experiments in which different samples of lard are used can be justly compared only when the linoleic acid contents have first been determined.

(4) *Comparison of the potencies of raisin seed oil and of linseed oil*

Raisin seed oil, which has only comparatively recently appeared on the market, is a by-product of the wine industry. A sample of material of Californian origin was contributed by Messrs W. J. Bush and Co., Ltd. for a biological test. This sample of raisin seed oil proved to have I.V. 122·4. It contained 93·5 % of fatty acids, the I.V. of which was 126·7, with 1·07 % of unsaponifiable matter. On bromination no ether insoluble bromide was obtained, and the percentage of linoleic acid was therefore calculated to be 38·6, no evidence of the presence of linolenic acid having been obtained.

The linseed oil used was that already described (p. 2166) as having a content of about 26 % linoleic, and about 44 % linolenic, acid.

Biological test. Four litter-mate rats received for 35 days 5 drops (about 0·08 g.) daily of the raisin seed oil, four received the same amount of linseed oil, and

Table VI. *Total wt. increase and healing of skin symptoms in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given linseed oil or raisin seed oil or no supplement*

Litter no.	Sex	Linseed oil 5 drops (0·08 g.) g.	Raisin seed oil 5 drops (0·08 g.) g.	No supplement negative controls g.	Linseed oil 5 drops (0·08 g.)	Raisin seed oil 5 drops (0·08 g.)	No supplement negative controls
4075	♂	39	52	10	+	+ +	θ
	♂	.	31	.	.	+ +	.
	♀	16	18	.	+ +	+ +	.
4076	♂	28	.	0	+	.	θ
	♀	17	12	5	+	+ +	θ
Av.		25	28	5			

three received no supplement. The average total weight increases in the period were 28, 25 and 5 g., respectively. All the four rats receiving raisin seed oil, and one rat receiving linseed oil, showed complete healing of the ankles in the period; the other three rats receiving linseed oil showed partial healing; the three receiving no supplement showed no healing (see Table VI).

A slight superiority of raisin seed oil over linseed oil seems therefore to be established. The result harmonizes well with the other results obtained and confirms the marked inferiority of linolenic to linoleic acid, since the linseed oil sample had a large content of linolenic acid, but somewhat less linoleic acid than the raisin seed oil.

(5) *Test of the potency of the methyl ester of the docosahexaenoic acid isolated from cod liver oil by Farmer & Van den Heuvel*

There is a mass of evidence in the literature showing that cod liver oil, although ineffective in curing the skin lesions produced by a fat-free diet, is effective in promoting weight increase. Thus Sinclair [1929–30] found that this oil, added as 10% of the otherwise fat-free diet of rats, did not cure scaliness, whereas 1% of lard was effective. Graham & Griffith [1930–31] reported a daily dose of 9–12 drops of cod liver oil as ineffective while wheat germ oil and lard cured well. Burr *et al.* [1930–31] gave 2–5 drops of cod liver oil daily and found weight increase renewed though the scaly tail was not cured. McAmis *et al.* [1929] found the rate of weight increase of fat-deprived rats much improved when 0·02 g. cod liver oil was added to the daily ration. These results would indicate the absence of linoleic acid from cod liver oil and the presence of other unsaturated acids able to promote weight increase but unable to alleviate the skin lesions [Burr *et al.*, 1930–31]. From the figures given by Banks *et al.* [1933] for the iodine values of the unsaturated acids derived from the depot fat of rats which had received a fat-free diet supplemented with cod liver oil, it is clear that when cod liver oil was given, highly unsaturated acids were laid down in the depot fat.

The composition of cod liver oil was investigated by Guha *et al.* [1930] who examined the methyl ester fractions of the unsaturated acids obtained by distillation under a pressure of 1 mm. If it is assumed that the iodine value of the fraction containing 18 carbon atoms is entirely due to the presence of oleic and linoleic acids, the proportion of linoleic acid would correspond with a content of about 12% in the original oil. The fraction containing 18 carbon atoms formed 18–32% of the total acids and absorbed from 2·8 to 4 atomic proportions of hydrogen. There was, however, no actual identification of linoleic or linolenic acid, and the same values could be obtained from mixtures of isomeric acids. In view of the absence of healing of the skin lesions when large doses of cod liver oil are added to the fat-free diet, it is difficult to believe that any appreciable amount of linoleic acid can be present in the oil [cf. Burr *et al.*, 1930–31].

Farmer & Van den Heuvel [1938, 2] have recently pointed out that, in all fractionations of the methyl esters of cod liver oil previously carried out, changes occurred which pointed to the occurrence of alterations in structure during distillation. After distilling the esters, even at a pressure of 0·1 mm., evidence of the presence of conjugated double bonds was obtained whereas there was no evidence of their presence before distillation. Much polymerization also occurred and the fractions isolated consisted of constant boiling mixtures. Farmer & Van den Heuvel [1938, 1] have, therefore, used the method of molecular distillation in which pressure was reduced to 0·001 mm. In this way, from 950 g. unsaturated acids, 225 g. of an acid containing 22 carbon atoms and 6 double bonds was isolated, no conjugated double bonds being present in the molecule

[Farmer & Van den Heuvel, 1938, 2]. The fraction containing 18 carbon atoms, when hydrogenated, gave pure stearic acid; the value for the hydrogenation number indicated that it contained 2·7 double bonds, so that in composition it approximated more nearly to linolenic than to linoleic acid.

A sample of the methyl ester of the C_{22} hexaenoic acid was contributed by Dr Farmer and was used for a biological test.

Biological test. The methyl ester of the docosahexaenoic acid had a strong fishy odour and was much disliked by the rats. It also showed in a high degree the property already mentioned (see p. 2164) of destroying the upper layers of the skin about the mouth, wherever it was allowed to come into contact with them, though no damage appeared to be caused to the mucous membrane of the mouth or intestinal tract. By intimate admixture with the daily yeast dose its consumption was satisfactorily secured.

Two rats received a daily dose of 5–8 drops (0·06–0·1 g.) of the material for 35 days, in which period the weight increase was 21 and 24 g. Such weight increase much improved the appearance of the animals, making it difficult to decide whether there was or was not some small improvement in the skin lesions; the ankles were, however, still rough and quite unhealed at the end of the period (see Table III). After the 5 weeks' experimental period the two animals were maintained on the same diet, without addition of the daily dose of acid, for another 14 days, on the chance that cure of the skin symptoms might continue and become more convincing as had been found to be the case with linoleic acid, but this did not occur. One rat, however, added another 5 g. of weight and the other 12 g. in this period. The behaviour of the rats was therefore in marked contrast with that of rats receiving linoleic acid, and there seem to be adequate grounds for distinguishing between the effects of the highly unsaturated acids of cod liver oil, which enable body fat to be laid down, and those of linoleic acid, which in addition to promoting weight increase, produce rapid amelioration and final cure of skin lesions.

(6) *Tests for potencies of chaulmoogra oil and chaulmoogric acid, methyl arachidate and 9:10:12-trihydroxystearic acid*

Chaulmoogra oil. The sample of oil used had I.V. 101·3. One rat received 10 drops (about 0·16 g.) daily for 13 days, but it lost weight and the oil appeared to be actually toxic (see Table III).

Chaulmoogric acid was prepared from chaulmoogra oil. It had M.P. 65·5° and I.V. 91·1. The same rat as above received 0·2 g. daily for 5 days; the material was badly consumed but appeared quite inactive, a result in agreement with that of Turpeinen [1938].

Methyl arachidate. In view of Turpeinen's work on the activity of arachidonic acid, it was of interest to see whether any evidence of activity could be obtained which would indicate the unsaturation within the body of arachidic to arachidonic acid. From arachis oil a fraction of saturated acid M.P. 72–75° was prepared, consisting mainly of arachidic acid. This was converted into the methyl ester. Owing to the high melting point the material would probably be badly absorbed in the biological test.

Biological test. One rat received 0·3 g. daily of the material for 15 days during which period it lost 9 g. and showed no alleviation of symptoms. The result was regarded as completely negative (see Table III).

9:10:12-Trihydroxystearic acid. The acid was prepared by oxidizing ricinoleic acid with $KMnO_4$; crystallized from alcohol it melted at 131–134°.

Biological test. One rat received 0·2 g. daily of the material for 35 days; it lost 4 g. and showed no improvement in skin symptoms. Like the ricinoleic acid tested by Turpeinen, it was thus completely inactive (see Table III).

Summary

1. The principal symptoms of the deficiency disease described by Burr & Burr as affecting rats maintained on a complete diet devoid only of fat were reproduced.

2. The efficacy of various materials adequately to supplement the fat-free diet was studied. Resumption of weight increase and healing of skin symptoms were the criteria used.

3. A rough method of estimating quantitatively the rate of healing of skin lesions is described. The importance of using some additional criterion more specific than weight increase is stressed.

4. The efficacies of methyl linoleate and methyl linolenate were compared; methyl linoleate was found much more potent and lasting in action than methyl linolenate, which possessed perhaps no more than one-sixth of the potency of methyl linoleate.

5. Various oxidation products of linoleic and linolenic acid were prepared and tested. Mixtures of α- and β- and of γ- and δ-tetrahydroxystearic acids in daily doses of 0·2 g. were completely ineffective in promoting weight increase or curing skin lesions in rats. Dioxidostearic acid was similarly inactive.

The hexahydroxy-derivatives, linusic and *iso*linusic acids, given in daily doses of 0·2 g., failed to benefit skin lesions but promoted a small but definite weight increase nearly equal to that evoked by administration of methyl linolenate in a daily dose of 0·08 g. All these oxidation products were solid substances of high melting point and were certainly absorbed only to a very limited extent by the rat's intestine.

6. The potency of a fraction of the unsaturated acids from lard was compared with that of a similar fraction from linseed oil and was found to be about the same, both as regards promotion of weight increase and healing of skin lesions.

7. A similar comparison was made of linseed oil with raisin seed oil; the latter was found slightly the more potent by both criteria. The linoleic acid contents of these two materials and of the fractions of linseed oil and lard were not greatly dissimilar but the linseed oil and its acids both contained 40% to 50% linolenic acid in addition. These results therefore confirm the superior potency of linoleic to linolenic acid.

8. The methyl ester of the docosahexaenoic acid isolated by Farmer and Van den Heuvel from cod liver oil was tested and found to be potent in promoting weight increase but to have little or no action in curing skin lesions.

9. Chaulmoogra oil and chaulmoogric acid, methyl arachidate and 9:10:12-trihydroxystearic acid were also tested and found inactive.

10. It is concluded that the ability of unsaturated fatty acids to supplement a fat-free diet in promoting weight increase is not necessarily associated with ability to heal skin lesions.

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